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## An ascorbate-reducible cytochrome b561 is localized in macrophage lysosomes

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### Abstract

Cytochromes b561 (Cyts b561) are a family of intrinsic membrane proteins involved in ascorbate-mediated transmembrane electron transport. The chromaffin granule Cyt b561 (CGCytb) is believed to transport electrons donated by extravesicular ascorbate (ASC) across the membrane to intravesicular monodehydroascorbate (MDA) supporting catecholamine synthesis in neuroendocrine tissues. Another isoform, the duodenal Cyt b561 (Dcytb), was reported to have ferric reductase activity, possibly facilitating intestinal iron uptake. Herein, a new Cyt b561 homologue, LCytb (for lysosomal Cytb561) was found expressed in the late endosomal–lysosomal membrane. LCytb shared high sequence similarity with CGCytb (45% identity) and Dcytb (42% identity). Moreover, four heme-coordinating His residues, and putative ASC and MDA binding sites were highly conserved. Recombinant LCytb exhibited an ASC-reducible b-type Cyt absorbance spectrum with  $\alpha$ -band maximum at 561 nm in the spectrum of the reduced protein. Northern blots and Western blots revealed that LCytb was predominantly expressed in lung, spleen, thymus, testis and placenta. In situ hybridization and immunofluorescence studies further demonstrated that the protein was expressed in the alveolar macrophages of the lung, in the white pulp of the spleen, widespread in the thymus, and in the Sertoli cells of the testis. Sequence analysis indicated the presence of a (DE)XXXL(LI)-type signal in the C-terminal of the protein, predicting a late endosomal–lysosomal subcellular localization. This localization was confirmed by double labeling experiments in RAW264.7 and 293 cells, stably transfected with LCytb.

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### 1. Introduction

Chromaffin granule Cyt b561 (CGCytb) is an intrinsic membrane protein expressed in neuroendocrine tissues. CGCytb is believed to function as an electron carrier transporting electrons from extravesicular ASC to intravesicular MDA to recycle ASC, supporting catecholamine synthesis [1]. It has only recently become evident that CGCytb is a member of a newly described protein family, ubiquitously present in almost all living kingdoms [2]. Using the CGCytb protein sequence, genome analysis has revealed the presence of multiple Cyt b561

isoforms in many species, with, for example, five isoforms in mice and four in *Arabidopsis thaliana* [2,3]. Although very little is known about any of the other Cyt b561 isoforms, sequence conservation suggests that they all have the structure motifs of Cyts b561, i.e. six putative transmembrane domains, four His residues which coordinate two heme molecules responsible for electron transport, and two putative binding sites involved in binding of ASC and MDA [2,4–6]. The sequence conservation suggests that all members of the Cyt b561 protein family are likely involved in ASC-mediated transmembrane electron transport. However, the localization and importance of the Cyt b561 isoforms other than CGCytb remain to be determined.

The so-called duodenal Cyt b isoform (Dcytb, or Dcybrd1) was localized in the mouse duodenal apical brush-border membrane [6]. Dcytb shared between 45% and 50% similarity to other members of the Cyt b561 family [6]. Subtractive cloning

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and expression of the Dcytb gene in *Xenopus* oocytes revealed that the gene encoded a protein with ferric reductase activity [6]. Dcytb-mediated reduction of ferric iron to ferrous iron is likely to facilitate iron absorption in the duodenum by DCT1 (Divalent cation transporter 1)/Nramp2 (Natural resistance-associated macrophage protein 2) [7,8]. However, Gunshin et al. recently reported that knockout of Dcytb had little or no impact on body iron stores, even in animals with low iron diet. This indicates that the role of Dcytb is far from clear [9,10].

Another Cyt b561 homologue, encoded by the gene 101F6, is localized in a 120-kb critical tumor deletion region (found in lung and breast cancers) of human chromosome 3p21.3 [11]. Forced-expression of wild-type 101F6 in human lung cancer cell lines significantly inhibited tumor cell growth by induction of apoptosis and alteration of cell cycle processes [12]. Moreover, intratumoral injection of Ad-101F6 significantly suppressed the growth of tumor xenograft and inhibited experimental lung metastases in nu/nu mice, but the underlying mechanism remains unknown [12].

As the Cyts b561 constitute a poorly characterized family of membrane proteins, herein we investigated the properties and the cellular and subcellular localization of another member of this protein family identified by genome analysis. Our results demonstrate that this Cyt b561 was localized predominantly in the late endosomal–lysosomal membranes and the protein has therefore been named LCytb.

## 2. Materials and methods

### 2.1. Plasmid constructs and yeast transformation

Standard PCR methods were used to amplify the gene (accession number: NM\_201351) encoding a putative new Cyt b561 from total mouse kidney RNA. Primers were designed to include *EcoRI* and *SpeI* restriction sites for cloning into the pESC-His expression vector (Stratagene, La Jolla, CA), downstream of the GAL10 Gal-inducible promoter. For transformation, yeast cells (strain YPH499, ura3-52 lys2-801amberade2-101ochre trp1-Δ63 his3-Δ200 leu2-Δ1) were grown in YPAD medium (Stratagene, La Jolla, CA) and transformation was performed according to the manufacturer's instructions. Transformed cell lines were selected on SD dropout medium lacking His (SD-His). For the induction of protein expression, overnight cultures were grown in SD-His and were transferred to 450 ml of synthetic Gal minimal medium containing 2% (w/v) Gal and supplemented with 1 mM ASC.

### 2.2. Plasmid constructs, cell culture and transfection

The mouse macrophage-derived cell line, RAW264.7 (ATCC) was cultured in DMEM medium containing 100 units L<sup>-1</sup> penicillin, 100 units L<sup>-1</sup> streptomycin, 10% FBS at 37 °C under 5% CO<sub>2</sub>. 293 cells derived from primary human embryonic kidney (GIBCO, Invitrogen Corporation) were cultured in the same condition as the RAW264.7 cells, except that they were supplemented with 0.1 mM non-essential amino acids. The LCytb gene was cloned into pcDNA3.1-vector (Invitrogen Life Technologies) with *XbaI* and *EcoRI* restriction sites, and transfected into the RAW264.7 and 293 cells using the FuGENE-6 transfection reagent (Roche Applied Science). The stably transfected cell clones were screened with G418 1 mg ml<sup>-1</sup> for RAW264.7 and 400 μg ml<sup>-1</sup> for 293 cells for 2 weeks.

### 2.3. Yeast microsomal membrane preparation and stripping

Yeast cells transformed with the LCytb gene or empty pESC-His vector were cultured overnight or longer until the OD<sub>600</sub> reached 0.6–0.8. The yeast microsomal membrane fraction was prepared as reported previously [13]. To

remove loosely bound proteins and proteins trapped inside sealed membrane vesicles, membranes were 'stripped' as follows: membrane fractions were suspended in 10 ml storage buffer, after addition of 0.05 ml 5% (w/v) Triton X-100 and 5 mM ASC, mixed vigorously and incubated on a rocker for 15 min at room temperature. Then 10 ml of 2 M KCl was added, and the fraction was incubated on the rocker for another 15 min. After addition of 20 ml nanopure H<sub>2</sub>O the fraction was again incubated for 15 min on the rocker. Membranes were collected by centrifugation at 75,000×g for 75 min at 4 °C, resuspended in storage buffer (25 mM MES-Tris, pH 6.8, containing 1% (w/v) glycerol), and stored at –85 °C until use. Protein concentration was determined using a modified Lowry method with BSA as the standard [14].

### 2.4. Animal organ microsomal membrane and cell lysate preparation

Animal organs (Pel-Freez biologicals, Rogers, AR) were homogenized in 10 volumes of ice-cold homogenization buffer (50 mM Tris–HCl pH 7.0, 150 mM NaCl, 1 mM ASC, 2 mM EDTA, 1 mM PMSF, 1 μg ml<sup>-1</sup> each of leupeptin, aprotinin, pepstatin A, antipain, and chymostatin). Debris and unbroken cells were removed by centrifugation at 5000×g for 10 min, the supernatant was centrifuged at 75,000×g for 75 min at 4 °C to collect the microsomal membranes, and pellet was resuspended in storage buffer. Stripping of the membranes was performed as described for yeast membranes.

Spleen and lung tissues were homogenized in liquid nitrogen, and kept in Munro's buffer (10 mM HEPES, pH 7.2, 3 mM MgCl<sub>2</sub>, 40 mM KCl, 5% glycerol, 0.2% NP-40, 5 mM DTT, 1 mM AEBSEF, 10 mg/ml Leupeptin and Complete™ EDTA free protease inhibitor cocktail (Roche Diagnostics)) for 15 min on ice. After centrifugation for 10 min at 13 000×g, the supernatants were kept for Western blot analysis. 293 cells transfected with LCytb or pcDNA3.1-empty vector were cultured until the confluence reached 80%, the cells were collected and rinsed with ice-cold PBS. Then the cell lysate was prepared with lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 100 mM Tris–Cl pH 7.4, 5 mM DTT, 1 mM PMSF, 1 μg ml<sup>-1</sup> each of leupeptin, aprotinin, pepstatin A, antipain, and chymostatin). The sample was centrifuged at 5000×g for 10 min; the supernatant was stored at –80 °C until use. Alveolar macrophages were prepared according to Holt's methods [15], the macrophages lysate was prepared as described for 293 cell.

### 2.5. Measurement of Cyt b absorption spectra

Absorption spectra of membrane-bound Cyt b in stripped yeast microsomal membrane vesicles were determined from oxidized and reduced spectra recorded in dual wavelength-mode (between 500 and 600 nm and reference at 601 nm; SLM-Aminco DW2000 spectrophotometer updated by OLIS Inc., Bogart, GA), in 2 ml samples with 1 nm slit-width, 0.5 nm s<sup>-1</sup> scan rate, and under continuous stirring. First, Cyts were oxidized by addition of ferricyanide (0.1 mM, FeCN) and the fully oxidized spectra were recorded. Then, ASC-reduced (25 mM ASC) and ASC + dithionite-reduced (after addition of Na-dithionite crystals) spectra were obtained at room temperature. When improvement of the signal to noise ratio was needed, multiple scans were averaged. In calculations, reduced minus oxidized difference spectra and 30 mM<sup>-1</sup> cm<sup>-1</sup> as molar extinction coefficient at 561 nm were used. The protein concentration was 2 mg ml<sup>-1</sup> throughout the experiments.

### 2.6. Northern blot analysis

A nylon (+) membrane blotted with 20 μg of total RNA in each lane from each mouse organ was purchased from Seegene, Seoul, Korea. Northern blots were performed according to the manufacturer's protocol. The template for the LCytb probe consisted of the nucleotides from 570 to 662 bp of the mouse LCytb cDNA, corresponding to the first 93 bp nucleotides of the coding sequence. The single strand DNA probes were synthesized and labeled with <sup>32</sup>P-dCTP by PCR with antisense primer only. The membrane was hybridized with <sup>32</sup>P-labeled DNA probes at 58 °C for 16 h, and subjected to autoradiography for 24–72 h.

### 2.7. Antibody generation, purification and western blots analysis

Antibodies were generated in rabbit against a C-terminal peptide ([Cys]-KRPDPGALTDQRQPLLHDRE) of mouse LCytb. After screening the sera for

the presence of LCytb-specific antibodies against yeast recombinant LCytb, antibodies were affinity purified using Sulfalink Coupling Gel (Pierce Biotechnology, Rockford, IL) according to the manufacturer's procedure. Membrane proteins were resolved by SDS-PAGE electrophoresis, using 12% acrylamide gels and transferred onto polyvinylidene difluoride membranes (PVDF, Bio-Rad, Hercules, CA). Each well was loaded with 15 µg samples for animal tissues and 1 µg sample for yeast recombinant LCytb. Prior to loading, samples were incubated at 37 °C for 30 min to denature the sample. Boiling was proved resulting in aggregation of the LCytb and preventing the protein from entering into the gel. LCytb antibodies were used in a 1:200 dilution, and protein-antibody complexes were detected by horseradish peroxidase-conjugated secondary antibodies (ECL detection kit, Amersham-Pharmacia Biotech, Piscataway, NJ). For peptide N-glycosidase F (PNGase F) digestion, aliquots of samples were digested with PNGase F according to manufacturer's instruction (New England Biolabs Inc.), after denaturing for 30 min at 37 °C in denaturing buffer.

## 2.8. Immunohistochemistry

Mouse organs removed from anesthetically killed animals were immediately fixed in ice-cold buffered 4% paraformaldehyde for 48 h and cryoprotected in 30% sucrose/0.1 M PBS for 48 h. The organs were sectioned into 10 µm sections, and then air-dried and stored at 4 °C until use. Sections were boiled in citrate buffer (0.01 M, pH 6.4) with a microwave oven for 15 min for antigen retrieval. Sections were incubated with the primary antibody for 2 h at RT or overnight at 4 °C, and were incubated with the secondary antibody for 1 h at RT. The sections were mounted with Gel Mount™ Aqueous Mounting Medium (Sigma-Aldrich).

For immunocytochemistry, the cells cultured on cover glass were fixed with ice-cold methanol for 5 min. For double-labeling with Lysotracker, the cells were incubated with 100 nM Lysotracker Red DND-99 (Molecular probes, Inc) in DMEM without serum for 30 min at 37 °C, then rinsed with PBS and fixed in ice-cold buffered 4% paraformaldehyde for 10 min. The cells were treated with 0.5% Triton X-100 for 15 min to increase their permeability. The cells were labeled with primary antibodies for 2 h and secondary antibody for 1 h at RT. The dilution was 1:50 for rabbit-anti-mouse LCytb, rat-anti-mouse MAC-3 and rat-anti-mouse Lamp2 (BD Biosciences, San Diego, CA), was 1:60 for CY2 labeled donkey-anti-rabbit and CY5 labeled donkey-anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA). The samples were observed under a fluorescence microscope (Nikon Eclipse TE300), or a confocal microscope (Olympus FV500).

## 2.9. In situ hybridization (ISH)

Sense and anti-sense cRNA probes were synthesized with the DIG RNA labeling kit (SP6/T7) (Roche, Indianapolis, IN). A nucleotide in 93 bp, the same region as used in the northern blot, was cloned into the plasmid pSPT18/19 as a template to generate cRNA. ISH was performed on cryosections (10 µm) with the SureSite® II system (Novagen, EMD Biosciences) according to the manufacturer's protocol. The sections were incubated with denatured sense or anti-sense cRNA probes (500 ng ml<sup>-1</sup> in hybridization buffer) at 50 °C for 18 h. The hybridized probes were visualized using anti-digoxigenin Fab fragments (dilution 1:500, Roche, Indianapolis, IN) and BCIP/NBT substrate, and observed under a Nikon Eclipse TE300 microscope.

## 2.10. RT-PCR

Total RNA was prepared from lung and alveolar macrophages with TRIzol® reagent (Invitrogen life technologies) according to the manufacturer's protocol. Two micrograms of RNA was used to prepare the cDNA. The following primers were used in the PCR experiments: LCytb, left: 5'-ATGGCTTCAG-GATGGTTT-3', right: 5'-TCATTCCCTGTATGCAA-3', yield a product of 726 bp, beta-actin, left: 5'-CTGTATCCCTCCATCGTG-3' right: 5'-TACTCTGCTGCTGATCCA-3', yield a product of 1000 bp. LCytb was amplified for 27 cycles and beta-actin was amplified for 23 cycles. The PCR products were separated in 1% agarose gel and taken picture with Gel Doc 1000 system (Bio-Rad).

## 3. Results

### 3.1. LCytb is an ASC-reducible Cyt b561

A protein-protein BLAST search of the NCBI protein database, using the bovine CGCytb sequence (NCB accession: NP\_031831) as a query, revealed the presence of five Cyt b561 homologs in the mouse genome. In addition to the mouse CGCytb, these are Dcytb [6], 101F6 [12,16], and two proteins with yet unknown function, Cyb561d1 (NCB accession: XP\_485295) and a hypothetical protein (NCB accession: NP\_958739). The latter is characterized in this study. Based on its subcellular localization in the late endosomes and lysosomes (see below), we named the new putative Cyt b561 as LCytb.

Multiple sequence alignment of mouse Cyts b561 revealed that LCytb shared high sequence similarity with CGCytb (45% identity over 211 amino acids), Dcytb (42% identity over 217 amino acids), 101F6 (29% identity over 136 amino acids) and Cyb561d1 (28% identity over 236 amino acids). LCytb was also highly conserved among different mammals, with 92% identity to rat putative LCytb (NCB accession: XP\_342023), 84% identity to human putative LCytb (NCB accession: AAH47710), and 83% identity to the chimpanzee putative LCytb (NCB accession: XP\_508473). Secondary structure prediction suggested that LCytb is an intrinsic membrane protein containing six consensus transmembrane domains (Fig. 1). Furthermore, LCytb has four conserved His residues most likely coordinating two heme molecules, and two putative binding sites for ASC and MDA. The N- and C-terminal ends of the protein were predicted to be localized in the cytoplasm by TMpred [17].

To identify whether LCytb is really a b-type Cyt, mouse LCytb was expressed in yeast. Spectroscopic analysis using microsomal membrane fractions from the transformed yeast cells demonstrated the presence of an ASC-reducible b-type Cyt (Fig. 2). In agreement with earlier observations [13], no ASC-reducible Cyts was detected in the microsomal membrane fraction from yeast cells transformed with the empty vector. The α-band of the reduced Cyt b is asymmetrical, with a maximum near 561 nm and a shoulder near 556–557 nm. The level of ASC reduction is about 52% of the maximal reduction obtained with Na-dithionite. The results therefore demonstrate that LCytb is indeed an ASC-reducible b-type Cyt.

### 3.2. Expression of LCytb

To contribute to our understanding of the physiological function of LCytb, we determined its organs and tissues distribution and its subcellular localization. In Northern blot analyses, the single strand DNA probe of LCytb hybridized with two mRNA bands, one of 3.3 kb, ubiquitously expressed in all organs tested, and another of 3 kb presented primarily in lung, spleen, thymus, testis, placenta (17.5 d.p.c.), small intestine, and stomach (Fig. 3). Comparing the density of the bands, the highest expression levels of LCytb were observed in lung, spleen, thymus, testis and placenta (17.5 d.p.c.). In





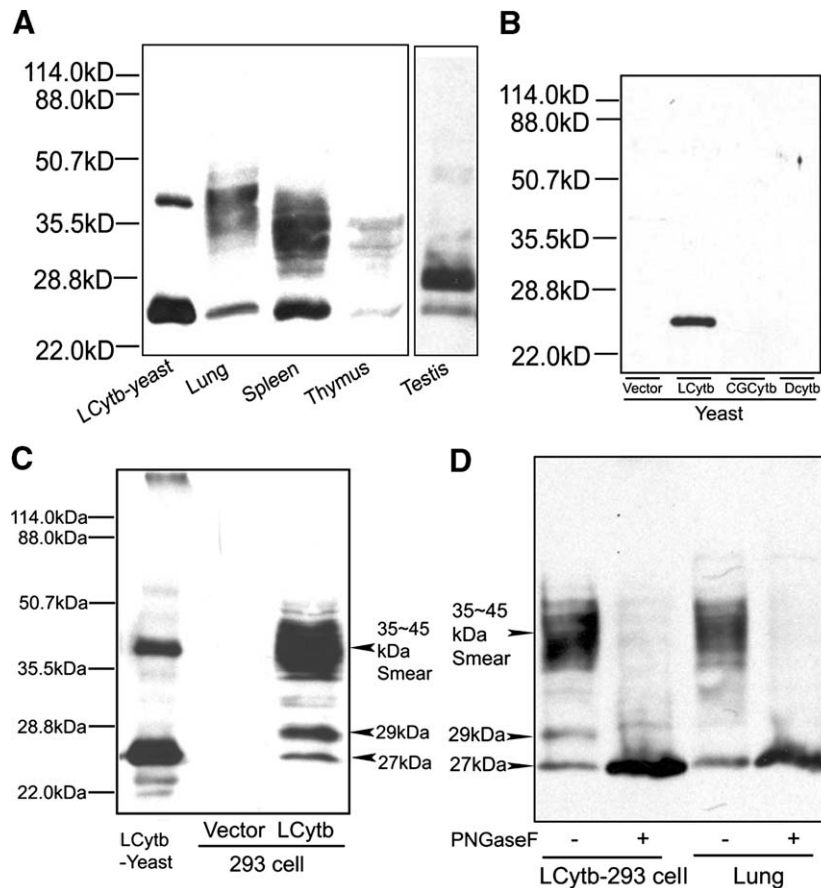


Fig. 4. Western blot analysis of the expression of LCytb protein. (A) Expression of LCytb in microsome membrane fractions of lung, spleen, thymus and testis was detected with an affinity purified C-terminal peptide antibody. Yeast recombinant mouse LCytb was used as a positive control. (B) The specificity of the antibody was assayed with fresh prepared membrane fractions of yeast transformed with mouse LCytb, CGCytb, Dcytb, or vector only. (C) Expression of LCytb in lysates of 293 cell transfected with LCytb or vector only. (D) Lysates of LCytb-transfected 293 cell and microsome membrane fractions of lung were treated with PNGase F to check the possibility of glycosylation.

might result from protein aggregation after repeated freezing and thawing of the sample, since there was little if any of this band detected in fresh sample (Fig. 4B). The affinity-purified antibody did not cross-react with any of the other mouse Cyts b561 when expressed in yeast, demonstrating that it was isoform-specific (Fig. 4B). No or little LCytb protein could be detected in microsome membrane preparations from other mouse organs such as brain, heart, liver and kidney (results not shown). A smear of higher molecular mass bands (35–45 kDa) was also observed in the Western blots from membrane fractions of lung, spleen and thymus (Fig. 4A). In contrast to that, in the testis, a band of about 29 kDa was also detected (Fig. 4A). In the cell lysate of LCytb-transfected 293 cells, a 27 kDa band, a 29 kDa band, and a smeared band at about 35–45 kDa were observed (Fig. 4C). These results demonstrated that these bands in the microsome membrane fractions of the various mouse organs, specifically resulted from the LCytb expression. Glycosylation of the intrinsic membrane protein DCT1/Nramp2 was reported to cause a smeared band at high molecular mass [18]. Hence, we speculated that glycosylation could also be the cause of the smeared bands observed in the organ membrane fractions. After the LCytb-transfected 293 cell lysate was treated with PNGase F, the band smear and the 29 kDa band

almost completely disappeared, while the 27 kDa band was strengthened, which suggested that the 29 kDa band and the band smear result from the glycosylation of the 27 kDa band (Fig. 4D). The effect of glycosylation was also confirmed by the treatment of lung membrane fractions with PNGase F, which resulted in the disappearance of the band smear and enhancement of the 27 kDa band.

### 3.3. Tissue localization of LCytb

To determine the tissue localization of LCytb, we performed ISH and immunofluorescence experiments. The anti-sense cRNA probe specifically labeled particular cell-types in the lung, spleen, thymus and intestine (Fig. 5A). Most of the positive cells in the lung are localized in the alveolus, and a smaller number of positive cells is located in the bronchia (arrow head in Fig. 5Aa). The positive cells in the spleen are mainly localized in the germinal center of the white pulp (Fig. 5Ac). In the thymus, the labeled cells were almost evenly distributed throughout the organ (Fig. 5Ae). In the intestine, a few cells in the lamina propria of the villi were specifically labeled (Fig. 5Ag).

Immunofluorescence studies, using the same C-terminal peptide antibodies used in the Western blots revealed similar



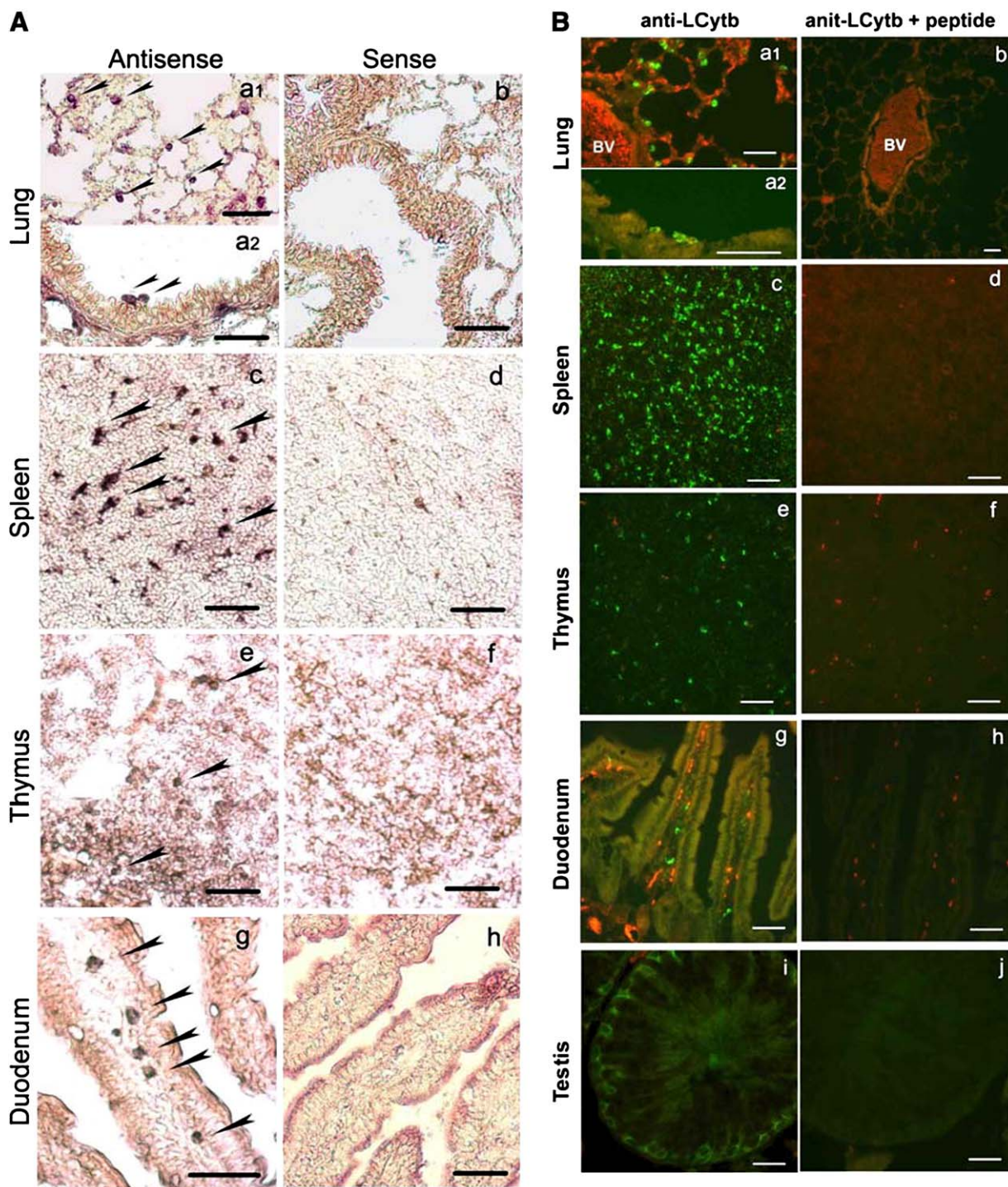


Fig. 5. Distribution patterns of LCytb and CGCytb in different mouse organs. (A) In situ hybridization (ISH) analysis of the LCytb expression in different mouse organs. Tissue sections from lung (a), spleen (c), thymus (e) and intestine (g) were labeled by the DIG-antisense cRNA probe, and sequential sections (b, d, f, h) were labeled by the DIG-sense cRNA probe as a control. (B) Immunofluorescence analysis of the localization of LCytb in different mouse organs. Sections, from lung (a), spleen (c), thymus (e), intestine (g) and testis (i), respectively, were probed with LCytb C-terminal peptide antibodies, and the antibody–protein complexes were labeled by CY2-donkey anti-rabbit antibody (Green). In the sequential sections from the corresponding tissue (b, d, f, h and j), antibody-cross reaction was blocked with the peptide demonstrating specificity of the antibody. The red color was from autofluorescence. a1 shows the lung alveolar region, and a2 shows the lung bronchial region. The results are representative of three experiments. The bar in the figure represents 50  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

distributions as observed with the ISH. Cells labeled in the lung were mostly evenly distributed in the alveolar region and appeared to be associated with the wall of the alveolus (green,

Fig. 5Ba1). A few labeled cells were localized in the bronchia (Fig. 5Ba2), while there were no cells labeled in the blood vessel (BV). Comparatively more cells were labeled in the spleen than

in the lung (Fig. 5Bc). As was also observed with the ISH, these were mainly localized in the germinal center of the white pulp, and a few cells were labeled in the red pulp. The positive cells observed in the thymus were more or less evenly distributed throughout the organ, with slightly more cells labeled in the medulla (Fig. 5Be). A few cells were specifically labeled in the lamina propria of the villi of the intestine (Fig. 5Bg). In the testis, the antibody very specifically labeled Sertoli cells (Fig. 5Bi). However, the fluorescence level in these sections was lower than that in other tissues. To demonstrate the specificity of the antibody, the antibody were preincubated with the C-terminal LCytl peptide before being added to the section, no labeling was found in these sections (Fig. 5Bb, d, f, h and j). A few labeled cells were also found in the cortex, but not in the medulla of the kidney (results not shown). Several other organs were also tested

for the presence of the LCytl protein but generally showed no specifically labeled cells (e.g. in brain, heart, liver). In general, the organ localization pattern of the LCytl protein obtained by immunofluorescence corresponds very well to the distribution of the LCytl mRNA obtained by ISH.

### 3.4. Identification of the cell type expressing LCytl

It is clear from the organ localization studies, that LCytl is expressed in specific cell types, such as the Sertoli cells in the testis, and in a well-defined cell type in the lung. As we suspected the positive cells in the lung to be macrophages, we simultaneously labeled lung sections with the LCytl antibody (Fig. 6A, a, d, green) and the macrophage marker Mac-3 (Fig. 6A, b, e, red) [19]. The merge of the images obtained

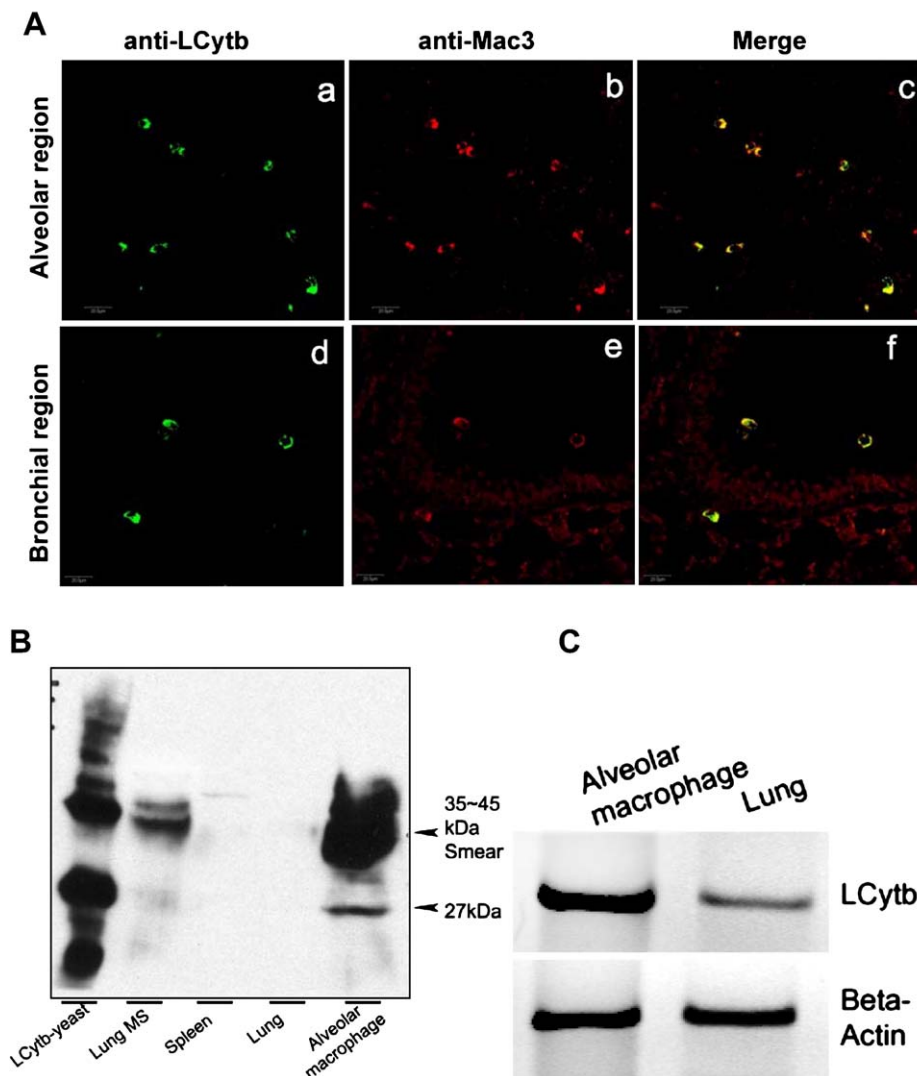


Fig. 6. Localization of LCytl to alveolar macrophages. (A) Double fluorescence labeling of lung sections with LCytl and macrophage marker antibodies. Lung sections were double labeled with LCytl antibodies (green) (panels a and d) and a macrophage marker, Mac-3 (red) (panels b and e). The pictures in the first row show the alveolar region, the pictures in the second row show the bronchial region. Panels c and f are the merges of a/b and e/f, respectively. (B) Western blot analysis of LCytl protein level in the lysate of alveolar macrophages (30  $\mu$ g), in the microsomal membrane fractions of lung (15  $\mu$ g), and in the homogenates of lung (30  $\mu$ g) and spleen (30  $\mu$ g). The yeast-recombinant mouse LCytl (1  $\mu$ g) was used as a positive control. (C) RT-PCR analysis to compare the LCytl mRNA level in alveolar macrophages and lung, beta-actin mRNA level was used as loading control. These results are representative of three experiments.



with the two antibodies (Fig. 6A, c and f) demonstrated that LCytb was specifically and exclusively expressed in the alveolar macrophages. A marker for type II pneumocytes, SP-B (Surfactant-associated protein B) was also applied to these

sections, but no overlap between SP-B and LCytb was observed.

In order to confirm the above results, alveolar macrophages were isolated, and the expression of LCytb protein in the lysate

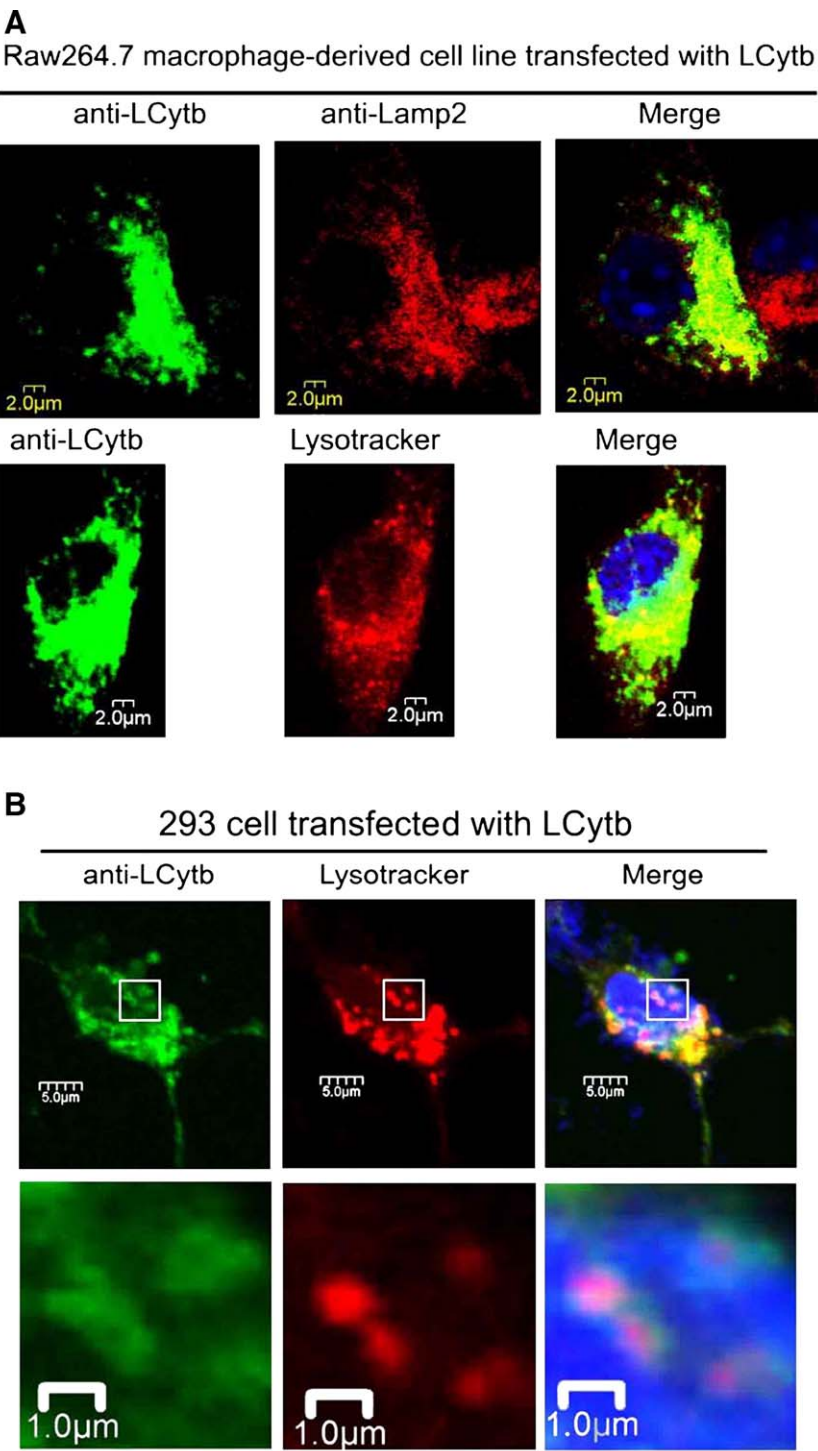


Fig. 7. Subcellular localization of LCytb in RAW264.7 and 293 cells. Double-labeling experiments analyzed the subcellular localization of LCytb in macrophage-derived cell lines, RAW264.7 (A) and 293 (B), stably transfected with mouse LCytb. (A) Top row: fluorescence in cells labeled with anti-LCytb antibodies (green), anti-Lamp2 antibodies (red), and the merge of these images. Bottom row: fluorescence in cells labeled with anti-LCytb antibodies, Lysotracker (red), and the merge of these images. (B) Top row: fluorescence in cells labeled with anti-LCytb antibodies, Lysotracker and the merge of these images. Bottom row: higher magnification of the boxed regions in the top row images. The blue signal is the result from nucleotide labeled by DAPI.

of alveolar macrophages was compared with that in the microsomal membrane fractions of LCytl-transfected yeast and lung, and that in the homogenates of spleen and lung (Fig. 6B). In the alveolar macrophages lysate, the antibody detected a 27 kDa band and a 35–45 kDa smear of bands, the band and the smeared bands were in the same pattern as that found in the microsomal membrane fractions of lung (Fig. 4). In contrast to alveolar macrophages lysate, the antibody detected very weak, if any, smeared bands at 35–45 kDa in the homogenates of lung and spleen (Fig. 6B). Considering that the intensity of the bands in the alveolar macrophages lysate was comparable to that in microsomal membrane fractions of LCytl-transfected yeast, and was much stronger than that in the microsomal membrane fractions of lung. The results suggested that LCytl is specifically expressed in alveolar macrophages. In accordance with the double labeling (Fig. 6A) and western blot results (Fig. 6B), the LCytl mRNA level is much higher in alveolar macrophages than that in lung (Fig. 6C).

### 3.5. Subcellular Localization of LCytl

We found that the C-terminal end of the mouse LCytl contained the peptide “DRQPLL” (Fig. 1, double-underlining). This motif was also highly conserved in the LCytl sequences from other mammals, but was absent from the other Cyt b 561 isoforms. “DRQPLL” is a putative dileucine-based sorting signal, that belongs to the (DE)XXXL(LI)-type signal family [20]. These signals target membrane proteins to the late endosomes and lysosomes, as well as to specialized endosomal–lysosomal compartments. The “DRQPLL” motif was located at the C-terminal of LCytl, 11 amino acid residues away from the TMD6, consistent with the general location of the (DE)XXXL(LI)-type signals [20]. Moreover, the Pro residue is also partially conserved in these signals [20]. The presence of this motif indicated that LCytl might be localized in the membrane of the late endosomes and lysosomes.

Since the expression level of the endogenous LCytl was low in cell lines, the subcellular localization of LCytl was studied in the RAW264.7 and 293 cells stably transfected with the mouse LCytl gene, using double-labeling fluorescence (Fig. 7). Two markers were used to determine the subcellular localization of LCytl, respectively, an antibody against Lamp2 (Lysosomal-associated membrane protein 2), and the so-called LysoTracker, a weakly basic amine selectively accumulating in cellular compartments with low internal pH. In the LCytl transfected RAW264.7 cell (Fig. 7A, upper panel), the fluorescence from the LCytl antibody (green) overlapped closely with that from Lamp2 (red). Similar results were obtained when the RAW264.7 cells were co-labeled with the LCytl antibodies and lysoTracker (red) (Fig. 7A, lower panel). To verify these results, the localization of LCytl was studied in the stably transfected 293 cell line (Fig. 7B). Fluorescence of the LCytl antibody and lysoTracker appeared located in discrete particles, and the two distributions patterns overlapped very well (Fig. 7B, upper panel). In a higher magnification of the merged images, it was observed that the lysoTracker accumulated into small particles, which appeared surrounded by the fluorescence from

the LCytl antibodies (Fig. 7B, lower panel). These results strongly suggested that LCytl is localized in the late endosomal–lysosomal membrane, and confirmed the localization predicted by the signal peptide.

## 4. Discussion

Cytochromes b561 are a family of intrinsic membrane proteins, involved in ASC-driven transmembrane electron transport. Although CGCytl, the first member identified in this family, has been studied for almost 30 years, there is little known about the other members of this protein family. In this work, a new Cyt b561 isoform, LCytl, was studied with Northern blot, Western blot, ISH and immunofluorescence.

LCytl has high sequence similarity with the other Cyt b561 isoforms (identity from 28% to 45%), it is also conserved in different mammals (identity from 83% to 92%). Moreover, LCytl has all of the structural elements found in Cyts b561. As other Cyts b561, LCytl has six predicted transmembrane domains, probably with the N- and C-termini in the cytoplasm (Fig. 1) [17]. Two fully conserved pairs of His residues (H47–H117 and H83–H156) are predicted to coordinate two heme molecules on opposite sides of the membrane respectively, conferring the property of transmembrane electron transport on the protein. The putative ASC and MDA binding motifs are conserved in LCytl, suggesting that LCytl could possibly utilize ASC and MDA as electron donor and acceptor [4]. Expression of the mouse LCytl gene in yeast demonstrated that this protein was indeed an ASC-reducible b-type Cyt, with a  $\alpha$ -band spectra almost identical to that of yeast recombinant-CGCytl and bovine CGCytl [13] (Fig. 2).

To start to understand the physiological role of this new Cyt b561, we performed tissue and cellular localization studies. Northern blot analyses, using a LCytl-specific probe corresponding to the 5'-terminus of the encoding region, demonstrated a 3.3 kb band ubiquitously present in all of the organs tested and a 3 kb band primarily present in lung, spleen, thymus, testis and placenta (Fig. 3). The two bands are possibly derived from alternative splicing. It is noteworthy that we were only able to detect the LCytl protein by immunofluorescence in the organs that showed the 3 kb band, which suggests that alternative splicing might result in the loss of the C-terminal epitope used to generate the antibody. The organ distribution pattern of LCytl is very different from those of two other Cyts b561, CGCytl and DCytl. CGCytl was mainly found in the adrenal medulla, the pituitary gland and other neuroendocrine tissues [21–23], while DCytl was mainly found in the duodenum and was possibly present in spleen, liver, brain, leucocytes, erythrocytes and neutrophils [6,24,25].

The purified polyclonal antibody generated against a C-terminal peptide detected three bands, including a 27 kDa band (present in the samples of lung, spleen, thymus and testis), a stronger smeared band (present in the samples of lung, spleen and thymus), and a 29 kDa band (present only in testis) (Fig. 4). Deglycosylation treatment with PNGase F indicated that the smeared bands and the 29 kDa band resulted from glycosylation of the 27 kDa protein. Therefore, the 27 kDa band could represent the premature form of LCytl, in the process of

maturation it is subject to different glycosylation, and the glycosylation for the protein in testis is different from that in other tissues. Because of differences in glycosylation, the protein might be endowed with different function or different compartmentalization, and this warrants further investigation.

To investigate the distribution of LCytb in tissues and organs, ISH and immunohistochemistry experiments were applied and yielded consistent results. In lung, LCytb was found expressed in the bronchial and alveolar region, double labeling with Mac-3 suggested that LCytb is expressed in a specific cell type in this organ, alveolar macrophages. In testis LCytb was specifically localized in Sertoli cells. The expression of LCytb in macrophages and Sertoli cells was confirmed in thioglycollate-elicited peritoneal macrophages, in the macrophage-derived cell lines, J774a.1 and RAW264.7, and in the Sertoli cell line MSC-1, using of RT-PCR and immunocytochemistry techniques (unpublished data). LCytb was also found expressed in the white pulp of spleen, in the lamina propria of the intestinal villi, and in thymus, however, the cell type expressing LCytb in these tissues is not known yet. It is remarkable that in an organ that expresses two distinct Cyt b561 isoforms, such as the intestine, expressing Dcytb and LCytb, these proteins are found in different regions. Dcytb is restricted to the brush border membrane [6], whereas LCytb is found in the lamina propria of the villi (Fig. 5g).

The C-terminal portion of LCytb contains a (DE)XXXL(LI)-type signal peptide, putatively targeting the protein to late endosomal–lysosomal membranes. This speculation is supported by double-labeling experiments, using stably transfected macrophage cell line, RAW264.7 and 293 cell line (Fig. 7). The localization of LCytb to the lysosomal membrane is remarkably consistent with the localization of other Cyts b561 that have been studied so far, to membranes separating an acidic and neutral compartment. CGCytb is localized in chromaffin vesicles with an acidic interior pH [26,27], Dcytb is localized in the plasma membrane of cells lining the acidic intestinal lumen [6], and a plant Cyt b561 (AtCytb1) has recently been localized to the vacuolar membrane, separating the acidic vacuolar content from the cytoplasm [28]. It has been argued that the existence of pH and charge gradients over the chromaffin vesicle membrane facilitates the electron transfer mediated by CGCytb from cytoplasmic ASC to MDA in the vesicle lumen [29]. It seems likely that similar pH gradients support the activity of the other members of this protein family.

As other Cyts b561, the electron donor for LCytb probably is cytoplasmic ASC, which is supported by the conserved ASC binding site in the loop toward the cytoplasm (Fig. 1) and by the reduction of yeast recombinant LCytb by ASC (Fig. 2). The electron acceptor for LCytb is not known yet, considering the sequence similarity, LCytb might have similar electron acceptor as its homologs. It could be MDA as that of CGCytb, in this case LCytb should have the ability to recycle ASC inside the late endosomes and lysosomes. Another possible electron acceptor could be the ferric ion as is likely for Dcytb. Ferric reductase activity for LCytb has indeed recently been demonstrated by expressing the protein in yeast cells [30]. It is notable that also CGCytb was recently reported to have ferric reductase

activity, and ferric reductase activity also was predicted for other isoforms of Cyts b561 [3,31].

Iron is an essential nutrient for almost all organisms, involved in a series of metabolic processes including electron transport, DNA synthesis, ATP production and cell division. Because of the low solubility of ferric ion, ferrous ion is the biologically available form, and ferric ion has to be reduced into ferrous ion for transporting by DCT1/Nramp2 that is localized in membranes of different cell types, such as apical membrane of duodenal enterocytes and endosomal membrane of macrophages [32,33]. For duodenal enterocytes, it probably is Dcytb that reduces the ferric ion into ferrous to facilitate the transport by DCT1/Nramp2 [6]. Dcytb displays ferric reductase activity when it is expressed in *Xenopus* oocytes and yeast cells, and its expression is up-regulated by iron-deficient diet and down-regulated by iron-replete diet in mice, similar as DCT1/Nramp2 [6,24]. For macrophages, an unknown ferric reductase localized in the endosomal membrane was suggested [7,8,34]. Therefore, LCytb could very well be a candidate for this ferric reductase facilitating Nramp2/DCT1-mediated transfer of iron into the cytoplasm.

In conclusion, LCytb, a new isoform of ASC-reducible Cyts b561, is described here. It is mainly expressed in the lung, spleen, thymus, intestine and testis. LCytb expressed specifically in the alveolar macrophages of lung, and also expressed in the Sertoli cells of testis. Signal peptide analysis and fluorescence studies suggested that LCytb is localized in the membranes of late endosomes and lysosomes.

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